



Summer 7-12-2018

Effect of Expansive Force on Mesenchymal Stem Cells Isolated From the Mid-Palatal Suture of Mice

Samaneh Mojarrad

University of Pennsylvania, mj.samaneh@gmail.com

Follow this and additional works at: https://repository.upenn.edu/dental_theses



Part of the [Dentistry Commons](#)

Recommended Citation

Mojarrad, Samaneh, "Effect of Expansive Force on Mesenchymal Stem Cells Isolated From the Mid-Palatal Suture of Mice" (2018). *Dental Theses*. 30.

https://repository.upenn.edu/dental_theses/30

This paper is posted at ScholarlyCommons. https://repository.upenn.edu/dental_theses/30

For more information, please contact repository@pobox.upenn.edu.

Effect of Expansive Force on Mesenchymal Stem Cells Isolated From the Mid-Palatal Suture of Mice

Abstract

Understanding the mechanisms by which craniofacial sutures respond to mechanical force is essential for improving orthodontic treatment strategies. However, the innate ability to regenerate bone from calvarial stem cells is still unknown. Therefore, we have initiated a study to isolate cells from the mid-palatal suture of mice. The aim of our study is to evaluate mesenchymal stem cell (MSC) characteristics of cells isolated from the mid-palatal suture and their ability for osteogenic differentiation when subjected to cyclic tensile force in vitro. A total of 10, 6-week old male C57BL/6 mice were used to obtain mid-palatal suture cells. Cultured cells were evaluated for MSC markers using flow cytometry and their potential for multi-lineage differentiation was evaluated using Alizarin Red S, Oil Red O, and Toluidine Blue staining. Cultured cells were subjected to cyclic tensile force with 15% elongation and a frequency of 0.5 Hz for 2 hours on the Flexcell-FX5000 tension system. Both stretched and control cells were cultured in osteogenic medium and the effect of tensile force on osteogenic differentiation was evaluated using Western Blot analysis and Alizarin Red S staining. Our results showed that mid-palatal suture cells formed colony-forming-units (CFU-F), expressed MSC-markers CD73, CD90, CD105, and Sca-1, and were negative for hematopoietic markers CD34 and CD45. In addition, these cells showed multi-lineage differentiation to osteogenic, chondrogenic, and adipogenic cell lines. Western blot analysis showed an upregulation in expression of osteoblastic markers, including ALP, OCN, and RUNX2 in the stretch group compared to control which was confirmed by a marked increase in extracellular matrix deposit in the stretched group using Alizarin Red S staining. In summary, our findings show that cells isolated from the mid-palatal suture of mice have MSC characteristics in vitro and that cyclic mechanical tensile strain can promote osteogenic differentiation through an upregulation of osteogenic markers and an increase in production of mineralized matrix.

Degree Type

Thesis

Degree Name

MSOB (Master of Science in Oral Biology)

Primary Advisor

Songtao Shi

Keywords

MSCs, Cyclic Tensile Force, Mid-Palatal suture, Gli1

Subject Categories

Dentistry



University of Pennsylvania Dental Medicine

Effect of Expansive Force on Mesenchymal Stem Cells Isolated from the Mid-Palatal Suture of Mice

Samaneh Mojarrad

A Dissertation in Orthodontics

Presented to the Faculty of Penn Dental Medicine in

Fulfillment of the Requirements for the Degree of Master of Science in Oral Biology

2018

Supervisor of Dissertation

Songtao Shi, DDS,MS,PhD

Chair and Professor

Department of Anatomy and Cell Biology

Graduate Group Chairperson

Chun-Hsi Chung, BDS,DMD,MS

Chauncey M.F Egel Endowed Chair

Associate Professor of Orthodontics

Dissertation Committee:

- Songtao Shi, DDS, MS, PhD. Chair and Professor , Department of Anatomy and Cell Biology.
- Chun-Hsi Chung, BDS, DMD, MS. Chauncey M.F Egel Endowed Chair, Associate Professor of Orthodontics
- Shuying Yang, MD, MS, Phd. Associate Professor. Department of Anatomy and Cell Biology
- Hyun Michel Koo, DDS, MS,PhD. Professor. Department of Orthodontics, Division of Pediatric Dentistry & Community Oral Health.

ACKNOWLEDGMENT

First, I would like to thank my mentor Dr. Shi, for giving me the opportunity to work under his supervision and guidance. As an undergrad student and a novice researcher, I became familiar with Dr. Shi's research and tremendous contributions to the field of "Stem cell and Tissue Regeneration". To have the opportunity to work with you and learn from your expertise has truly been a dream come true!!

Thank you Dr. Chung for your continuous support and encouragement, and for granting me acceptance into the combined Orthodontics/MSOB program. I will forever be grateful for the opportunity.

I would like to thank my committee members, Dr. Yang, and Dr. Koo for being gracious with their time to be part of my thesis committee.

Thank you Dr. Wenjing Yu, for your continuous guidance throughout my research. You have taught me so much this past year, but most importantly, I have learned to be patient and to never give up.

Lastly, I would like to thank my family and friends for their unconditional love, support, and encouragement in every step of my educational journey.

ABSTRACT

Effect of Expansive Force on Mesenchymal Stem Cells Isolated from the Mid-Palatal Suture of Mice

Samaneh Mojarrad

Songtao Shi, DDS, MS, PhD

Understanding the mechanisms by which craniofacial sutures respond to mechanical force is essential for improving orthodontic treatment strategies. However, the innate ability to regenerate bone from calvarial stem cells is still unknown. Therefore, we have initiated a study to isolate cells from the mid-palatal suture of mice. The aim of our study is to evaluate mesenchymal stem cell (MSC) characteristics of cells isolated from the mid-palatal suture and their ability for osteogenic differentiation when subjected to cyclic tensile force in vitro. A total of 10, 6-week old male C57BL/6 mice were used to obtain mid-palatal suture cells. Cultured cells were evaluated for MSC markers using flow cytometry and their potential for multi-lineage differentiation was evaluated using Alizarin Red S , Oil Red O, and Toluidine Blue staining. Cultured cells were subjected to cyclic tensile force with 15% elongation and a frequency of 0.5 Hz for 2 hours on the Flexcell-FX5000 tension system. Both stretched and control cells were cultured in osteogenic medium and the effect of tensile force on osteogenic differentiation was evaluated using Western Blot analysis and Alizarin Red S staining. Our results showed that mid-palatal suture cells formed colony-forming-units (CFU-F), expressed MSC-markers CD73, CD90, CD105, and Sca-1, and were negative for hematopoietic markers

CD34 and CD45. In addition, these cells showed multi-lineage differentiation to osteogenic, chondrogenic, and adipogenic cell lines. Western blot analysis showed an upregulation in expression of osteoblastic markers, including ALP, OCN, and RUNX2 in the stretch group compared to control which was confirmed by a marked increase in extracellular matrix deposit in the stretched group using Alizarin Red S staining. In summary, our findings show that cells isolated from the mid-palatal suture of mice have MSC characteristics in vitro and that cyclic mechanical tensile strain can promote osteogenic differentiation through an upregulation of osteogenic markers and an increase in production of mineralized matrix.

TABLE OF CONTENTS

ACKNOWLEDGMENT.....	ii
ABSTRACT.....	iii
CHAPTER 1:Introduction.....	1
CHAPTER 2:Materials and Methods.....	10
CHAPTER 3:Results.....	18
CHAPTER 4:Discussion.....	27
BIBLIOGRAPHY.....	31

Chapter 1

Introduction:

The skeleton is a load bearing structure able to respond to a variety of genetic and epigenetic factors. One important epigenetic factor is the mechanical environment, the stresses and strains, to which skeletal cells are subjected. Chondrocytes, osteoblasts and osteoclasts are constantly exposed to physical forces that modulate the cellular phenotype and gene expression during development and postnatal growth. The ability of bone and cartilage to respond to mechanical stress provides the foundation for many orthopedic and orthodontic procedures [1]. The use of mechanical force to correct dento-facial deformities was pioneered by Angell and Kingsley [2]. Orthopedic maxillary expansion was first described over 145 years ago by Angell in 1860 [3] [4].

Posterior crossbite in children with mixed dentition is reasonably common, occurring in 7.1 o/o of U.S. children aged 8 to 11. It usually results from narrowing of the maxillary arch [5]. Reduced transverse growth of the maxilla can lead to underdevelopment of the mid-face, sagittal problems, and posterior crossbite causing occlusal disharmony and functional problems involving breathing pattern anomalies [6] [7]. The most effective orthopedic treatment to increase maxillary transverse width is rapid maxillary expansion (RME) [4]. The mid-palatal suture, located between the maxillary bones in the palate, contains secondary cartilage that is highly responsive to various mechanical forces [1]. Sutural mechanical strains, caused by RME, triggers a biologic chain of events leading to new bone deposition in the mid-palatal suture [8]. Mesenchymal cells located on the

inner side of the cartilaginous tissue proliferate and differentiate into osteoblasts when the suture is expanded [8] [1] and therefore new bone formation is known to occur between the palatal bones in the suture [9].

Mesenchymal Stem Cells (MSCs):

Stem cells are clonogenic cells that have the capacity for self-renewal and multilineage differentiation [10] [11]. Stem cells can be divided into two main types; embryonic stem (ES) cells and adult stem cells [11]. Adult stem cells are often relatively slow-cycling cells that are able to respond to specific environmental signals. They either generate new stem cells or select a particular differentiation program [12].

MSCs, first described by Friedenstein et al. [13] [14], were originally found and isolated from the bone marrow, in 1970. They were referred to as colony forming unit-fibroblasts, and their capability to differentiate to various mesenchymal tissues gave rise to the concept of MSCs. These cells are among the most promising adult stem cells [15] and are defined as pluripotent cells with the ability to differentiate along osteogenic, chondrogenic, and adipogenic lineages [14].

In 2006, the International Society for Cellular Therapy (ISCT) proposed minimal criteria to define human multipotent MSCs. According to the ISCT criteria, MSCs must be adherent to tissue-culture-treated plastic when maintained in standard culture conditions. Additionally, MSCs must express CD105, CD73 and CD90 and lack the expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules.

Finally, MSCs must be able to differentiate to osteoblasts, adipocytes and chondroblasts in vitro [16] [15].

The differentiation of MSCs is controlled by several factors including biological factors, extracellular matrix (ECM), and mechanical stress [7]. The mechanical properties that influence differentiation of stem cells can be classified into two groups: intrinsic and extrinsic cues. The intrinsic mechanical cue indicates the property of the stem cell itself, which includes elasticity, stiffness, viscoelasticity, and adhesion. The extrinsic mechanical cue refers to properties of the extra-cellular matrix (ECM) or matrix. These intrinsic and extrinsic mechanical properties are closely related to each other. The ECM affects the intrinsic mechanical properties of the stem cells, which causes changes in the differentiation capability or lineage and vice versa [17].

Craniofacial Sutures; A Niche for MSCs of Craniofacial Bones:

Cranial and facial sutures are soft connective-tissue articulations between mineralized bones in the skull. As joints, sutures absorb and transmit instantaneous mechanical stresses upon either natural activities such as mastication or exogenously applied forces such as orthopedic loading . The niche is the in vivo microenvironment that regulates stem cell survival, self-renewal, and differentiation [18]. Adult stem cells are often localized to specific niches, where they utilize many of the external and intrinsic cues used by their embryonic counterparts in selecting a specific fate. Locating and analyzing stem cell niches are important steps in determining the key components of the environment that legislate differentiation commitments and stem cell regulation [12].

In 2015, in a study conducted by Zhao et al., using mouse craniofacial bones as a model, Gli1+ cells within the suture mesenchyme were identified as the main stem cell population for mesenchyme of most craniofacial sutures such as; the inter-palatal, maxilla-palatal, maxilla-premaxilla, and inter-maxilla sutures. Gli1+ cells expressed typical MSCs characteristics in vitro and were detectable only within the suture mesenchyme, mostly in the mid-suture region, but were absent from the periosteum, dura and osteocytes. Thus, indicating that craniofacial sutures provide a unique niche for MSCs [19]. Successively Maruyama et al., also identified and isolated a suture stem cell population that expresses high levels of Axin2. These Axin2-expressing cells were restricted to the suture midline and maintained their localization in the niches, and produced osteogenic descendants during skeletal development and homeostasis [20].

Cellular Response to Mechanical Stress In Craniofacial Sutures:

In the 19th century, a relationship between mechanical stress and bone formation was suggested by Julius Wolff [2]. He stated that mechanical stress was responsible for the architecture of bone and that bone remodels and adapts according to the mechanical demands that it has to withstand [2] [21]. Cells have mechano-sensory capabilities and the ability to convert mechanical signals into a biochemical response via mechanotransduction [21] [22]. Exogenous force applied to bone is transmitted as mechanical stress [23]. This mechanical stress is then transferred to the cellular level by

causing deformation in the extracellular matrix of suture cells [22], and initiating a cascade of signaling events within the cell which regulates their differentiation [2] [17] .

In recent years, both in vivo and in vitro methods have been used to study mechanical forces in craniofacial sutures [2]. The type, frequency, and magnitude of mechanical forces have all been shown to affect MSC differentiation.

➤ **Type of Force:**

The effect of four types of external mechanical signals have been studied on MSCs: fluid flow, compression, hydrostatic pressure and tension [24]. In regards to type of force, it has been convincingly demonstrated that overall tensile forces induce osteogenic differentiation (Simmons et al., 2003; Friedl et al., 2007; Qi et al., 2008, Diederichs et al., 2009) and compression forces induce chondrogenic differentiation (Huang et al., 2004; Campbell et al., 2006; Thorpe et al., 2012; Steward et al., 2014; Luo et al., 2015) [25] [26] . In a study directly comparing cyclic compression and tension, tension was found to regulate many osteogenic and fibroblastic genes, while compression enhanced many chondrogenic-related genes [24] .

➤ **Frequency of Force:**

Recently, effects of cyclic mechanical stimulation on osteogenic differentiation of human intraoral MSCs were analyzed by Lohberger et al. In this study, Reverse transcription quantitative real-time PCR revealed that 10% continuous cyclic strain (0.5 Hz) induced a significant increase in the mRNA expression of the osteogenesis-specific

markers type-I collagen, osteonectin, bone morphogenetic protein-2, osteopontin, and osteocalcin in osteogenic differentiated MSCs. Furthermore, mechanically stimulated groups produced significantly higher amounts of calcium deposited into the cultures and alkaline phosphatase (ALP) [27] [26].

➤ **Magnitude of Force:**

In 2011, Liu et al., conducted a study to establish the causal relationships between expansion force magnitudes, sutural separation, and sutural bone formation in the midsagittal suture of rabbits. Bone markers showed that sutural widths increased with increase in force magnitude. Sutural bone formation also increased with increasing forces up to 100 g, however, there was no difference between the 100-g and the 200-g groups. Successively, Jang et al. reported that the magnitude of cyclic tension may regulate MSC fate decisions, with myogenesis favored at high tensile strains while low tensile strains were more beneficial for osteogenesis of rabbit MSCs [28]. In addition, similar results were observed in a study by Liu et al. In this study, the suture cartilage response was compared when constant 10 g and 20 g continuous expansive forces were delivered to the mid-palatal suture of mice for a week. Their study showed that even though similar osteogenic differentiations were induced by the 10 g and the 20 g expansions, however, the lower expansion (10 g) promoted chondrocyte proliferation and induced a more preferable suture cartilage response pattern compared with the higher expansion (20 g), which just increased the cartilage matrix production [16] [29].

Expansive Force (aka Tensile Force) and the Mid-Palatal Suture:

For half a century the expansion of the mid-palatal suture, has been successfully used to correct dentofacial deformities and treat maxillary width deficiencies [30] . Therefore, the effect of force on maxillary sutures has been intensely studied, as the maxillary sutures are the key targets during orthopedic treatment of a narrow maxillary arch [2] .

The application of orthodontics forces to the suture has been shown to modify the normal configuration of the cartilage structure. Previously, Takahashi et al. (1996) reported the occurrence of a marked revision from cartilage to bone triggered by orthodontically applied expansional forces [31] . In this study, the cartilage at the mid-palatal suture in rats was examined as an experimental model. At the end of the experiment, most of the cartilaginous tissues were separated from each other and the mid-palatal suture was replaced by osteocalcein-positive intramembranous bone and fibrous sutural tissue. These results strongly suggest that the application of tensional force to the mid- palatal suture cartilage changed the phenotypic expression of osteochondroprogenitor cells in the secondary cartilage, indicating pathway differentiation from chondroblastic to osteoblastic [32] [31] .

It has been shown that osteoprogenitor cell number in sutures increase in response to tensile stress application, and their increase is proportional to both the duration and magnitude of applied force. However, the increase in number was more sensitive to the duration of stress than it was to the stress magnitude. The number of osteoprogenitor cells increased in response to high-magnitude tensile stress at the beginning of stress

application, and then decreased rapidly. Furthermore, osteoprogenitor cell number was equal to that in sutures exposed to low stress levels but for longer duration [2]. In addition, Zahrowski et al. studied the effects of force magnitude on osteoprogenitor cell activity during premaxillary expansion in 3-month-old male rats. It was concluded that an increase in force magnitude was correlated with increased numbers of labeled cells up to 100 gm, with decreased cell numbers at higher forces. The numbers of labeled cells at 200 gm were not significantly different from the controls [33]. Thus, supporting evidence from previous studies favoring lower magnitude tensile force in promoting osteogenic differentiation.

Furthermore, Kobayashi et al. evaluated the early cellular events evoked by expansional force on the mid-palatal suture of growing rats. In response to expansional force, the lateral cartilaginous layers moved away from each other and only mesenchymal cells distributed along the inner lateral side of the cartilaginous tissue, exhibited strong immunoreactivity for expressing proliferating cell nuclear antigen (PCNA). These results indicated that only mesenchymal cells located on the inner side of the cartilaginous tissue proliferate and differentiate into osteoblast [31]. Such findings were concurrent with prospect studies by Zhao et al. and Maruyama et al. providing information that sutures are a niche for MSCs.

Suture's uniqueness can be described as consisting of mesenchymal derived cells and their matrices in a confined environment ready to be loaded with different types of mechanical stimuli and therefore are a unique model for investigating the mechanical

modulation of biological growth [23]. Studies of how mechanical forces influence skeletal structure and function shed light on basic bone cell biology and help improve strategies for treating skeletal diseases [1]. Therefore, understanding the mechanisms by which craniofacial sutures respond to mechanical force is essential for improving orthodontic treatment strategies [30]. Recent studies have begun to uncover the nature of skeletal stem cells qualified for the more rigorous stem cell definition. In the calvarium, there is every expectation that the suture is the niche for stem cells which regulate calvarial bone development. However, stem cells of the calvarial bones have yet to be isolated, and their innate ability to regenerate bone is still unknown [20]. Due to these shortcomings and to obtain better insight into suture-MSCs, we have initiated a study to isolate mesenchymal stem cells from the mid-palatal suture of mice in order to evaluate their stem cell characteristics and ability for osteogenic differentiation when exposed to mechanical cyclic tensile (aka expansive force) *in vitro*.

Chapter 2

Study Design:

2.1. Objectives:

- Isolation and characterization of MSCs from the mid-palatal suture of mice
- Evaluate the effect of tensile force application on MSCs isolated from the mid-palatal suture in vitro
- Evaluate the presence of Gli1+ MSC population in the mid-palatal suture

2.2. Hypothesis:

- Isolated mid-palatal suture cells express MSC characteristics
- In vitro tensile force on mid-palatal suture MSCs will promote osteogenic gene expression and induce their osteo-inductive properties
- Gli1+ cells are present in the mid-palatal suture

2.3. Null Hypothesis:

- Isolated mid-palatal suture cells do not express MSC characteristics
- In vitro tensile force on mid-palatal suture MSCs will not promote osteogenic gene expression and will not induce their osteo-inductive properties
- Gli1+ cells are not present in the mid-palatal suture

2.4. Study Significance:

- To obtain insight into the cellular activity and the underlying mechanisms that drive bone formation when applying expansive forces with a RPE

- The identity and characteristics of suture stem cells responsible for craniofacial bone formation and regeneration are highly limited. To this date, there is no evidence of isolating suture stem cells from the mid-palatal suture and evaluating their response to mechanical tensile force. The current study is the first attempt to isolate MSCs from the mid-palatal suture and to evaluate the effect of tensile force on their osteogenic differentiation

- In contrast to other cranial sutures, evaluating tensile force on the mid-palatal suture is more convenient and does not require a surgical procedure to insert a metallic implant. Therefore, it is arguably the most convenient suture for studying interactions between mechanical tensile stress and MSC differentiation

- Due to their utility in uncovering genetic mechanism, mice represent an ideal model for studying the responses of mammalian craniofacial bones and sutures to mechanical forces

Material and Methods:

Experimental Animals:

A total of 10, 6-week old male C57BL/6 mice were used to obtain cells from the mid-palatal suture. Mice at this stage are in a growing phase and their first and second maxillary molars are fully erupted. All animals were euthanized in a CO₂ gas chamber and their death was confirmed with cervical dislocation. All animal work was performed using protocols approved by the Institutional Animal Care and Use Committee of University of Pennsylvania, Philadelphia PA.

Isolation and Culture of Suture-MSC:

After removing the palate from maxilla and removal of the soft tissue, the exposed palatal suture was excised along with 0.5 mm of adjacent structures on both sides. The suture tissue was minced into small pieces using a scalpel blade #15 and subjected to enzymatic digestion using an enzyme solution consisting of 3 mg/ml collagenase type I and 4 mg/ml dispase 1:1 ratio for 1h at 37°C. Later, three ml of Dex(-) solution was added to the digested tissue and centrifuged at 1500 rpm for 6 minutes. Suspended cells were then filtered through 70 micro millimeter strainer, seeded in 100 mm culture dishes (Genesee), cultured in alpha minimum essential medium (α -MEM, Invitrogen) supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine (Invitrogen), 55 μ M 2-mercaptoethanol (Invitrogen), 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen), and incubated in an atmosphere of 5% CO₂ at 37°C of temperature. After 24 hours, non-adherent cells were removed , and adherent cells were cultured for an additional 15 days with the above mentioned medium with the addition of Dex(+) at day seven. These adherent single colonies were passaged at passage one (P1) with frequent medium changes to eliminate potential hematopoietic cell contamination.

Flow cytometry analysis:

Flow cytometry analysis was used to determine the expression of mesenchymal and non-mesenchymal stem-cell surface markers of the isolated cells. Cultured suture mesenchymal cells of P1 were dissociated into single cells by treating the cells with 3 ml 0.05% trypsin/ 0.02% EDTA for 10 min at 37°C and vigorous pipetting. Cells were then

Centrifuged at 1500 rpm for 6 min at 4°C. A total of 0.2- 0.3 x10⁶ cells were resuspended in 100 µl of wash buffer per FACS tube/antibody. The following antibodies were used: anti-CD34, anti-CD45 (hematopoietic, non-MSC-associated markers), anti-CD73, anti-CD90, anti-CD105, anti-Sca1 (MSC-associated markers), and isotype-matched control (negative control) at a concentration of 1µg of primary antibody or isotype-matched antibody for 30-45 minutes on ice. Cells were then washed twice in 1 ml wash buffer, centrifuged at 1500 rpm for 6 min at 4°C, and resuspend in 500 µl of 2 % PFA (Wash Buffer : FACS Fixative => 1:1). All samples were analyzed with FACSCalibur and CellQuest software (BD Bioscience). PE conjugated antibodies to CD45, CD73, and CD105, were purchased from Thermo Fisher Scientific. PE conjugated antibodies to CD34 and anti-Sca1 were purchased from BD Bioscience. PE conjugated antibody CD90 was purchased from BioLegend.

***In vitro* Osteogenic Differentiation:**

P1 cells were plated onto 6-well plates at a density of 5·10³ cm² cells per well in the proliferation medium and allowed to reach confluency. The medium was then replaced with basic osteogenic differentiation medium including α-MEM (Invitrogen), 20% fetal bovine serum, 100 U/ml penicillin and streptomycin (Invitrogen), 2mM Glutamine, 10⁻⁸ M Dexamethasone sodium phosphate, 55 µM 2-ME, 0.1 mM L-ascorbic acid (Wako), 2 mM beta-glycerophosphate (Sigma-Aldrich). Medium was changed every three days.

Alizarin Red S Staining:

After 4 weeks of osteogenic differentiation, Alizarin Red S (Sigma-Aldrich) staining was performed to detect extracellular mineralization. Briefly, cells were fixed in 100% ethanol and stained with a 1% Alizarin Red S solution (pH 6.4). The red staining represents calcium deposits on terminal differentiated cells.

***In vitro* Chondrogenic Differentiation:**

P1 cells (4×10^5 cells/culture) were centrifuged in conical 15-ml polypropylene tubes in 1 ml of medium for 6 minutes at 1500 rpm. Cell pellets were treated with chondrogenic differentiation medium which was composed of DMEM (Gibco), 15% FBS, 1% Insulin Transferin Selenium + (BD Bioscience), 0.1 μ M Dexamethasone sodium phosphate (Sigma), 2 mM Sodium pyruvate (Sigma), 100 U/ml penicillin and streptomycin (Biosource), 0.1 mM L-ascorbic acid (Wako), 2 mM L-glutamine (Biosource), supplemented by 10 ng/ml Transforming Growth Factor- β 1 (TGF- β 1, R & D). The cultures were incubated at 37°C and 5% CO₂ for four weeks with medium changes of twice weekly. To prepare the cultures for the assessment of cartilage differentiation, the pellets were fixed with 4% PFA for 4h, decalcified in EDTA for 4h, and then dehydrated in ascending concentrations of ethanol, cleared in xylene and embedded in paraffin wax. Five μ m-thick sections were then made and stained by Toluidine Blue.

***In vitro* Adipogenic Differentiation:**

For adipogenesis, P1 cells were plated at 5×10^3 cm² in six well plates and cultured under adipogenic inductive conditions, in growth medium containing: α -MEM, 15% FBS, 0.5 mM isobutylmethylxanthine (Sigma-Aldrich) , 60 μ M indomethacin (Sigma-Aldrich), 0.5 μ M hydrocortisone (Sigma-Aldrich) , 10 μ g/ml insulin (Sigma-Aldrich), 100 U/ml penicillin and streptomycin (BioSource), 0.1 mM *L*-ascorbic acid phosphate (Wako), 2 mM L-glutamine (BioSource). The cultures were incubated for two weeks during which the medium was changed twice weekly. At 14 days post induction, the adipocytes were stained with Oil red O (Sigma-Aldrich) for lipid droplet.

Mechanical Stimulation:

The Flexcell FX-5000 Tension System (FX5K; Flexcell International Corp, Hillsborough, NC) was used to apply mechanical cyclic tensile stretch to the suture-MCSs. The Flexcell FX-5000 is a computer based system that uses a vacuum to strain cell adhered to the flexible silicone membranes (BioFlex plates; Flexcell International Corp) arranged in a format of six wells per plate with a total growth of 9.62 cm²/well and a membrane thickness of 0.05 mm. The deformation of the flexible membrane of the plates causes the attached cells to deform. Programming the magnitude, duration, and frequency of the negative pressure in the Flexcell apparatus creates the desired strain profiles. Suture MSCs were seeded onto the collagen type-1-coated BioFlex plates at a density of 4×10^4 cells/ well. When cultures reached approximately 70-80% confluence, undifferentiated MSCs were subjected to cyclic mechanical stimulation with equibiaxial

waveform with 15% elongation and a frequency of 0.5 Hz for 2 hours. Each cycle consisted of 10 second strain and 20 seconds relaxation. Control cultures were grown under the same condition but without the strain protocol. All cells were kept in an incubator during active stretching at 37°C in a humidified atmosphere containing 5% CO₂.

Western Blot Analysis:

Cells were lysed in M-PER mammalian protein extraction reagent (Thermo Fisher Scientific) with protease and phosphatase inhibitors (Roche), and then protein content was quantified using the Pierce™ BCA Protein Assay Kit (Thermo Scientific). 30 mg of protein was separated on an SDS-PAGE gel and transferred to nitrocellulose membranes (Millipore). The membranes were blocked with 1% non-fat milk (Santa Cruz), 4% BSA, and 0.05% Tween-20 for 1h and then incubated overnight with primary antibodies diluted in incubation buffer, according to the manufacturer's instructions. Antibodies for mouse alkaline phosphatase (ALP), runt-related transcription factor-2 (RUNX2), and osteocalcin (OCN) were purchased from Santa Cruz Biotechnology, Inc. The membranes were incubated for 1h in HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) diluted at 1:100,000 in incubation buffer. Immuno-reactive proteins were then detected by SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and BioMax film (Kodak).

Immunohistochemistry staining for Gli-1:

Isolated MSCs from the mid-palatal suture were seeded on two-chamber slides and cultured for 24-48 hours. After being washed in phosphate-buffered saline (PBS, pH 7.4) and fixed in 4% p-formaldehyde (PFA) for 10 minutes, the samples were washed twice with PBS, and permeabilized with 5% BSA/0.3% Triton-X100 for 30 minutes, and then incubated with 1:200 Gli-1 anti body overnight. Each sample was washed twice with PBS and incubated with second antibody (goat poly-clonal anti-rabbit IgG-Alexa 488, Invitrogen) for an hour. Finally, the nuclei were stained in DAPI (2 mg/mL) for 5 minutes.

CHAPTER 3

Results:

3.1. Isolation of Clonogenic Populations of Mid-Palatal Suture Cells

Cells isolated from the mid-palatal suture showed adherent clonogenic cell clusters of fibroblast-like morphology. Each colony originates from a single progenitor cell, which is called colony-forming unit-fibroblast (CFU-F) (Fig 3.1A). The cells within each colony are characterized by a typical fibroblast-like morphology (Fig 3.1B).

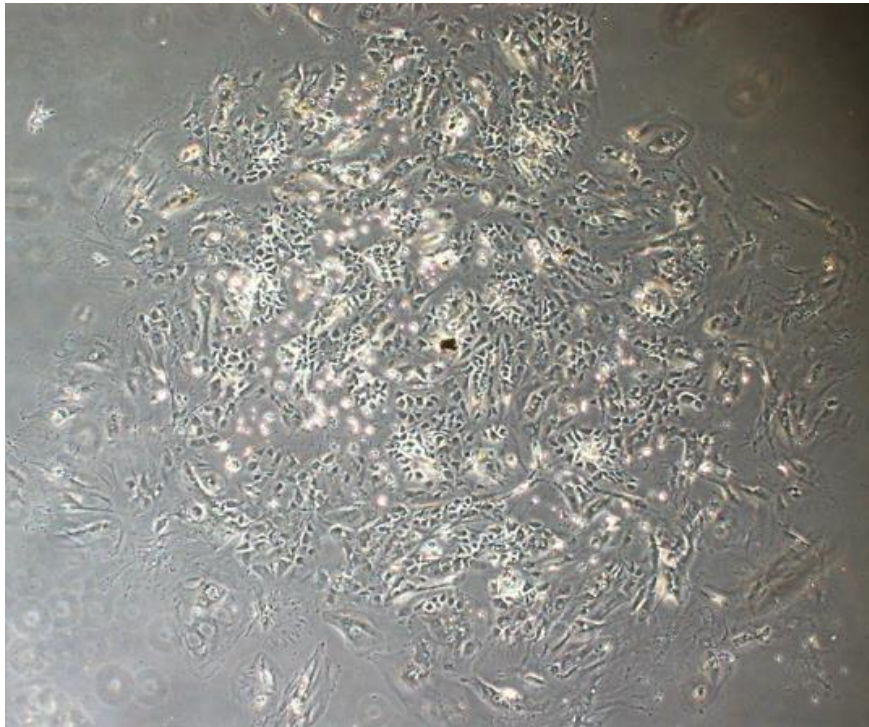


Figure 3.1A: Cells isolated from the mid-palatal suture showing formation of colony-forming unit-fibroblast (CFU-F)

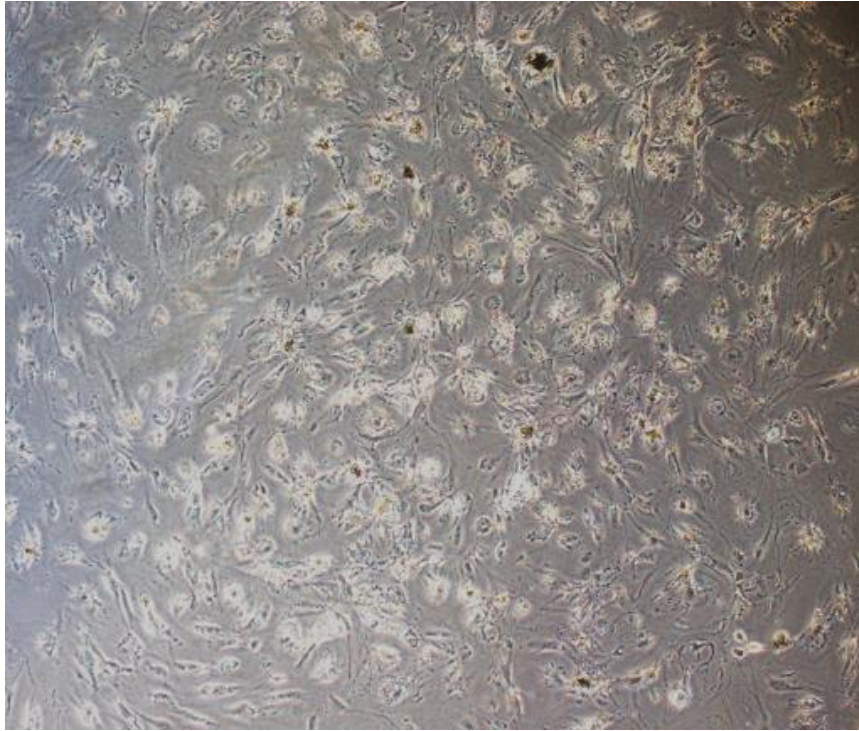


Figure 3.1B: Cells isolated from the mid-palatal suture showing adherent clonogenic cell properties and fibroblast-like morphology

3.2. Flow Cytometry

Flow cytometry was performed to determine the expression of mesenchymal and non-mesenchymal stem-cell surface markers of the isolated cells. Our results showed that isolated suture cells were positive for MSC-markers such as; CD73, CD90, CD105, and Sca-1 and negative for hematopoietic markers such as; CD34 and CD45 (Figure 3.2).

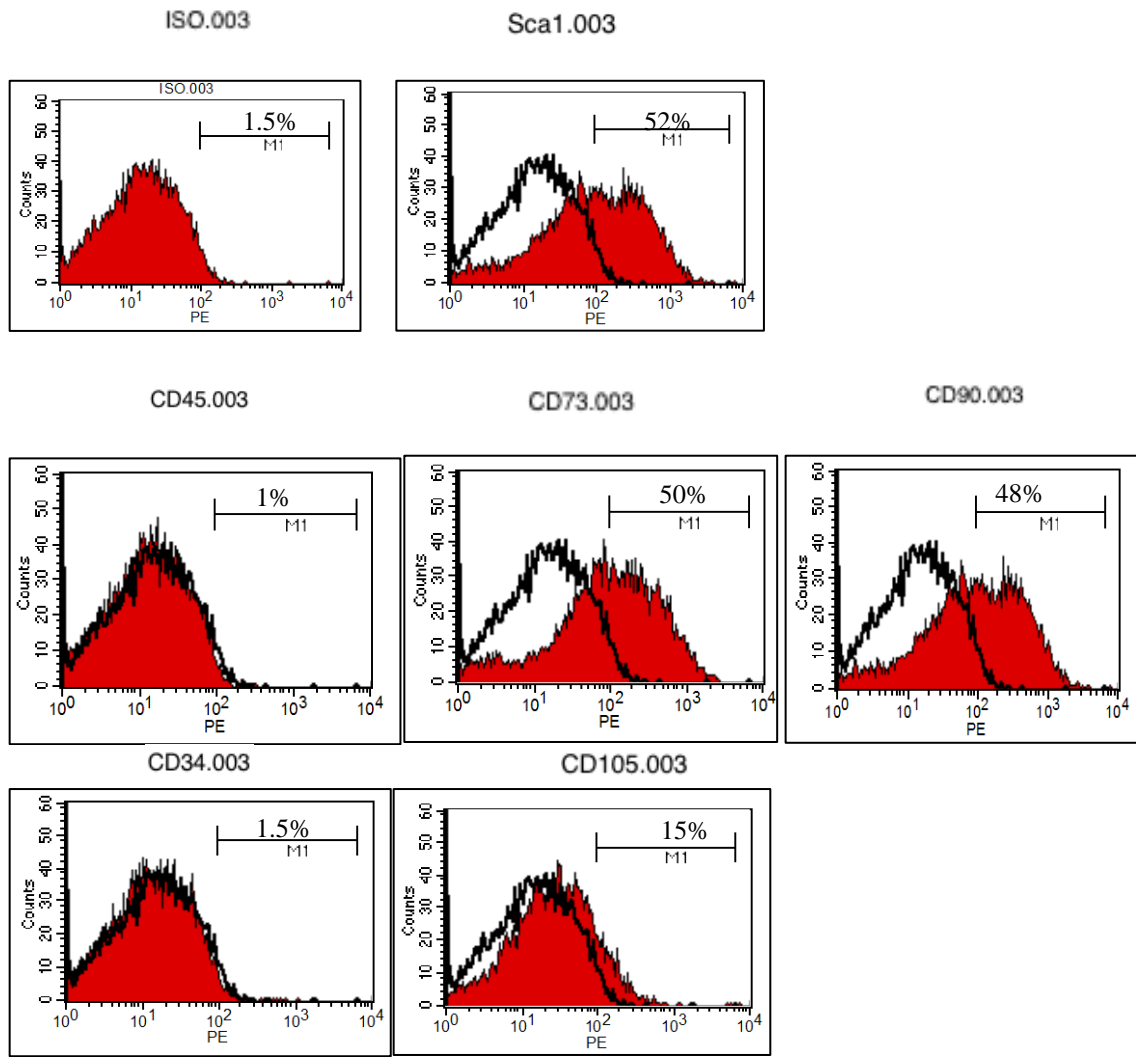


Fig 3.2: Isolated suture cells show positive expression for markers Sca1,CD73,CD105,and CD90 and are negative for CD34 and CD44.

3.3. Differentiation Potential of Mid-Palate Suture MSCs in Vitro

Osteogenic Differentiation: To investigate the potential of suture MSCs to differentiate into mineralizing osteoblasts, P1 cell cultures were induced to form mineralized matrix containing calcium deposits by the addition of culture media containing L-ascorbate-2-phosphate, dexamethasone and beta-glycerophosphate (osteogenic media). After 4 weeks in culture, suture MSCs demonstrated the capacity to form Alizarin Red- positive condensed nodules indicating calcium accumulation *in vitro* (Fig 3.3A)

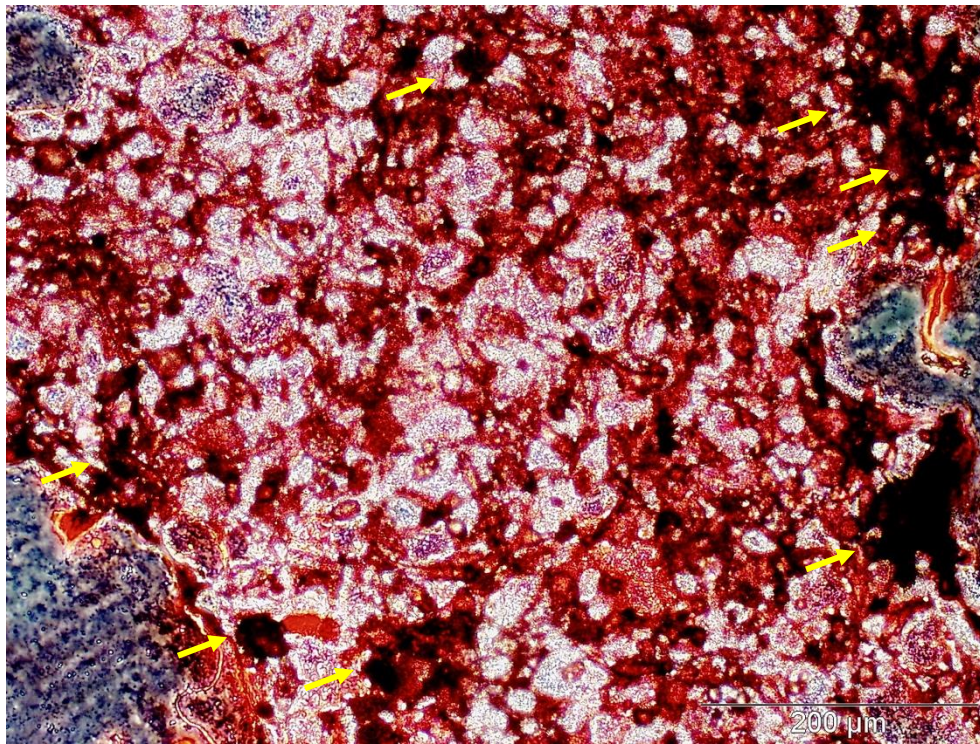


Fig 3.3A: Osteogenic differentiation of mid-palatal suture MSCs. Alizarin red staining showing mineralized nodule formation (yellow arrows) of MSCs after 4 weeks of induction with osteogenic medium.

Adipogenic Differentiation: P1 mid-palatal MSCs were assessed for their potential to differentiate into other cell lineages such as adipocytes. After 4 weeks of culture with an adipogenic inducing media, the cells were stained with Oil Red O stain, and showed positive lipid-laden droplets (Fig 3.3B).

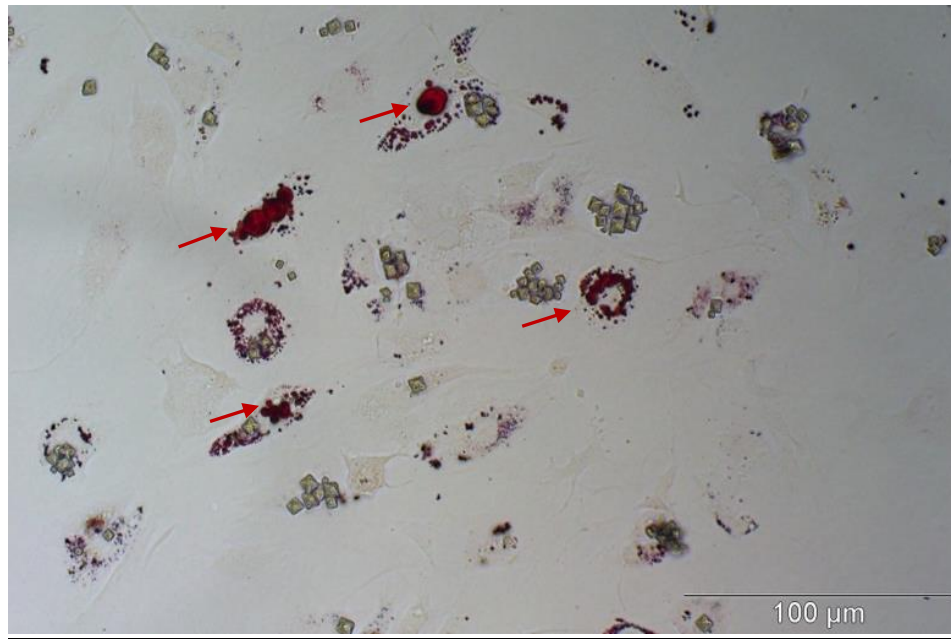


Fig 3.3B: Adipogenic differentiation of mid-palatal suture MSCs. Cultured MSCs formed Oil red O positive lipid clusters (red arrows) after 4 weeks of induction in the presence of adipogenic medium.

Chondrogenic Differentiation: P1 cells were also assessed for their potential to differentiate into chondrocytes. After 4 weeks of exposure to chondrogenic differentiation media, mid-palatal suture MSCs stained positive with Toluidine Blue (Fig 3.3C). Histologically, cell pellets showed characteristics of chondrogenic differentiation such as; round cells resembling hyaline chondrocytes, and formation of areas with cartilaginous lacunae (Fig 3.4D).

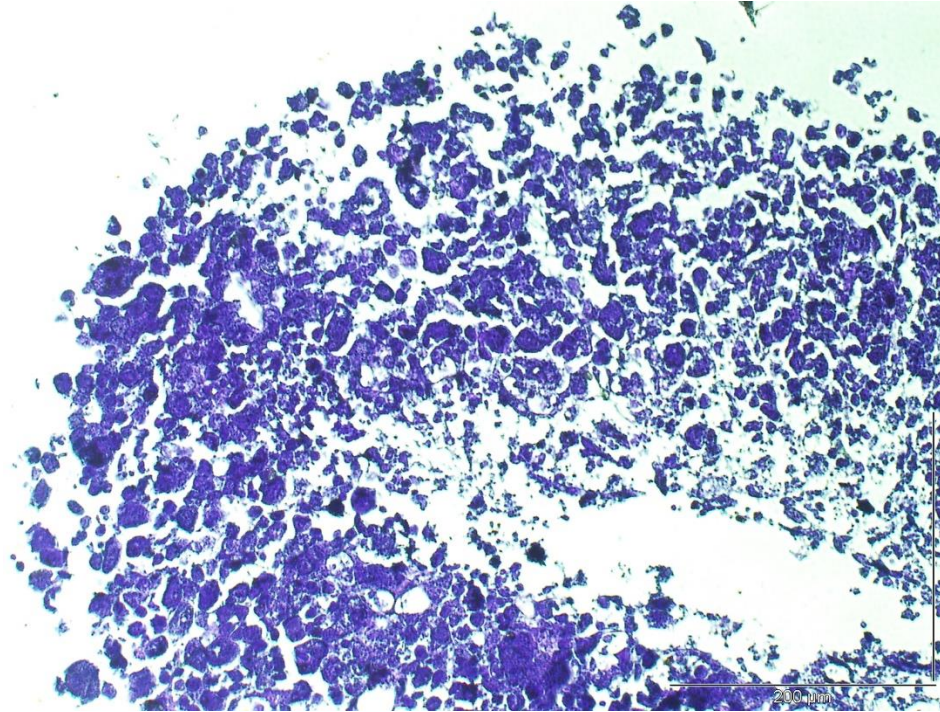


Fig 3.3C: Chondrogenic differentiation of mid-palatal suture MSCs assessed by toluidine blue staining of pellet sections.

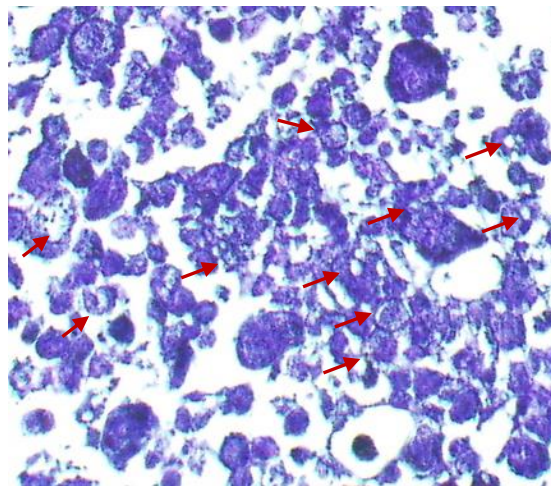


Fig 3.3D: Cell pellets showed characteristic of chondrogenic differentiation such as; round cells resembling hyaline chondrocytes, and formation of areas with cartilaginous lacunae (red arrows)

3.4. *In vitro* Tensile Force Application and Osteogenic differentiation:

To evaluate the effect of tensile force (aka expansive force) to promote osteogenic gene expression and induce osteo-inductive properties on isolated mid-palatal MSCs, undifferentiated cells were subjected to cyclic mechanical tensile stimulation with equibiaxial waveform with 15% elongation and a frequency of 0.5 Hz for 2 hours. Next, stretched and control cells were cultured with osteogenic medium and were evaluated for osteoblastic differentiation using Western Blot analysis and Alizarin Red S staining.

Western Blot Analysis:

Stretched and control cells were cultured with osteogenic medium for one week and then evaluated for expression of osteogenic markers. Western blot analysis showed an increase in expression of osteoblastic markers, including alkaline phosphatase (ALP), osteocalcin (OCN), and RUNX2 in the stretch group compared to control (Figure 3.4A).

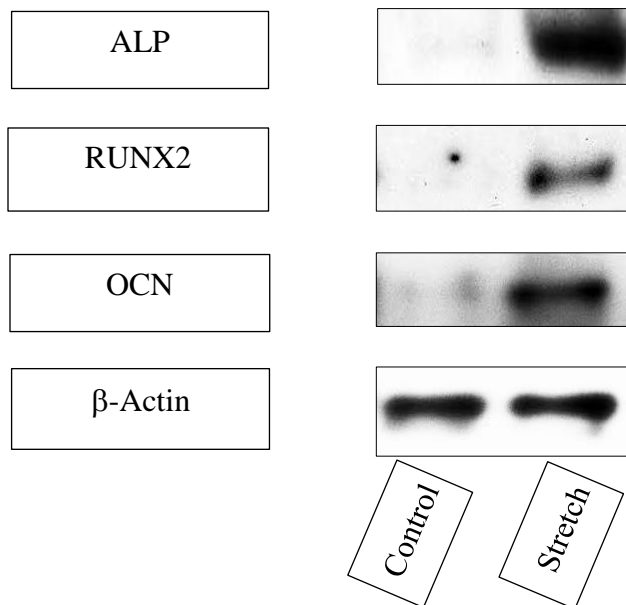


Fig 3.4A: Western blot analysis revealed increase in expression of osteoblastic markers, ALP, RUNX2, and osteocalcin in stretch group compared to control.

Alizarin Red S staining:

Four weeks after tensile force application, Alizarin Red S (Sigma-Aldrich) staining was performed to detect extracellular mineralization. Alizarin Red staining showed increase in extracellular mineralization in stretch group compared to control group. Quantitative analysis with Image J software showed 40% extracellular mineralization in stretched group compared to 17% in control group.

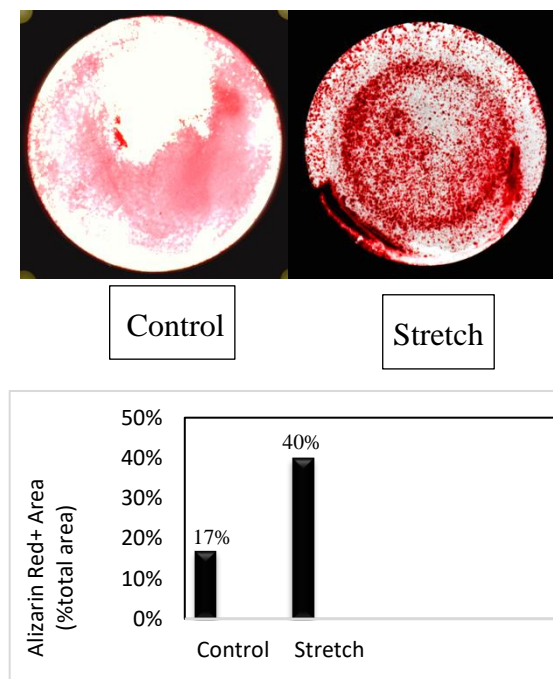


Fig 3.4B: Alizarin Red staining showed increase in extracellular mineralization in stretch group compared to control group.

3.5. Immunohistochemistry staining for Gli-1 cells:

In a recent study by Zhao et al, Gli-1 + cells were identified as the main MSC population in craniofacial sutures [19] . To investigate if Gli1 + cells were detectable in our cell culture we did indirect- IHC staining for Gli1 marker. As seen in figure 3.4, mid-palatal suture MSCs stained positive for expressing Gli1 marker. Quantitative analysis showed that 97% of the mid-palatal suture-MSCs expressed Gli1 markers.

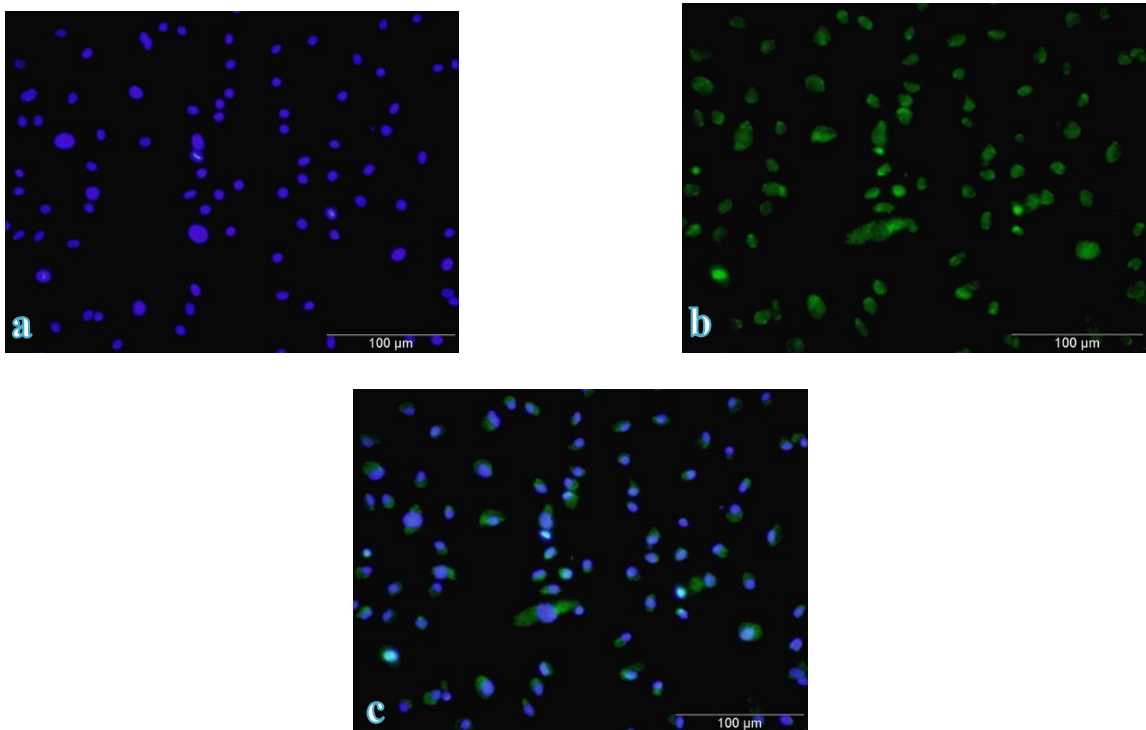


Fig 3.5: MSCs from mid-palatal suture expressing Gli1 marker. (a) Nuclear staining by DAPI (blue), (b) immunofluorescence staining of Gli1 marker (green), and (c) MSCs expressing Gli1 marker.

CHAPTER 4

Discussion:

Results from the present study showed that cells isolated from the mid-palatal suture of mice have MSC properties according to the criteria defined by the International Society of Cell Therapy (ISCT) [15] [16]. In our study, cells isolated from the mid-palatal suture showed adherent clonogenic cell clusters and colony-forming-units (CFU-F) with typical fibroblast-like morphology. Flow cytometry results revealed that the isolated suture cells were positive for MSC-markers such as; CD73, CD90, CD105, and Sca-1 but negative for hematopoietic markers such as CD34 and CD45. In addition, the isolated cells showed the ability for “multilineage differentiation”. Mid-palatal suture cells were able to differentiate into osteogenic, adipogenic, and chondrogenic cell lines. These results were similar to findings reported by Zhao et al and Maruyama et al, showing MSC properties of cells isolated from the sagittal suture in mice [19] [20].

For decades, mid-palatal suture expansion has been clinically used for maxillary transverse correction. MSC are known to be capable to differentiate into osteoblast-like cells, in response to physiological mechanical loads in vivo and in vitro [10, 14, 34]. The most widely used mechanical stimuli in vitro are cyclic stretch and fluid shear flow [35]. Therefore in the present study, we evaluated the effect of tensile force (aka expansive force) on osteogenic gene expression and its osteo-inductive properties on MSCs isolated from the mid-palatal suture of mice. Undifferentiated cells were subjected to cyclic mechanical tensile stimulation with equibiaxial waveform with 15% elongation and a frequency of 0.5 Hz for 2 hours. Western blot analysis showed increase in expression of

osteoblastic markers, including alkaline phosphatase (ALP), osteocalcin (OCN), and RUNX2 in the stretch (tensile) group compared to control. Our findings were similar to Zhang et al., who reported that human BM-MSCs exposed to tensile mechanical strain of 10% elongation and a frequency of 1 Hz, stimulated osteogenic differentiation by activating Runx2, followed by increased alkaline phosphatase (ALP) activity and mRNA expression of osteogenesis-related genes such as ; ALP, collagen type I, and osteocalcin [36]. Also, Friedl et al. reported that application of continuous tensile strain to human BM-MSCs significantly stimulated the expression levels of osteogenic marker genes such as collagen type 1A1, RUNX2, ALP, secreted phosphoprotein 1 (SPP1), and SPARC when analyzed by RT-PCR [37]. Zhao et al. and Jang et al., respectively reported an upregulation of alkaline phosphatase in MSCs isolated from rat-bone marrow (BM) and rabbit-tibia and femoral bones, when subjected to cyclic tensile force [19, 28]. In addition, Zhao et al. reported an increase in the mRNA expression of osteocalcin and RUNX2 of rat BM-MSCs when stimulated by cyclic tensile force of 3% elongation and 1 Hz frequency [19]. In studies examining the effects of tensile force, stem cells are typically seeded on a flexible membrane or within a matrix to which strain is applied. Some factors that have been varied amongst different studies include force magnitude, frequency, and application time. Therefore, results from separate studies are difficult to compare directly, but demonstrate that tensile force can induce osteogenic differentiation, although the magnitude of the tensile force applied varies between studies [38].

In our study, results from Alizarin Red staining showed significant increase in production of extracellular mineralized matrix in stretched group compared to control.

This result is in accordance to other reported studies by Lohberger et al., Ward et al., Simmons et al., and Weismann et al, which demonstrated an upregulation of mineralized matrix formation in MSCs subjected to mechanical cyclic tensile force [14, 27, 39, 40]. The production of mineralized matrix is considered a marker for terminally differentiated MSCs into osteoblast- like cells [14, 27, 41]. Therefore, mineral formation is an appropriate indicator in that mechanical stimulation accelerates the osteogenic differentiation of MSCs.

In 2015, in a study conducted by Zhao et al, Gli1⁺ cells within the suture mesenchyme were identified as the main stem cell population for mesenchyme of most craniofacial sutures indicating that craniofacial sutures provide a unique niche for MSCs [19]. In 2017, Shi et al. and colleagues provided evidence that the Gli1⁺ cells are a predominant source for osteoblasts throughout the life of a mouse. Their findings showed that embryonic Gli1⁺ cells give rise to essentially all osteoblasts in both fetal and postnatal skeleton. Thus, suggesting that Gli1 can be identified as a common molecular marker among most if not all mesenchymal progenitors destined to become osteoblasts in the mouse [42]. Therefore, lastly we evaluated if MCS from the mid-palatal suture of mice express Gli1 marker. Our results were similar to findings from Zhao et al. and Shi et al., showing that 97% of MSCs isolated from the mid-palatal suture of mice expressed Gli1 marker.

To this date, previous studies have only attempted to isolate MSC from sagittal suture of mice. To the best of our knowledge, our study was the first attempt to isolate and

characterize MSCs from the mid-palatal suture of mice and to evaluate their osteo-inductive properties in response to cyclic tensile force.

In contrast to other cranial sutures, evaluating tensile force on the mid-palatal suture is more convenient because it does not require a surgical procedure to insert a metallic implant for force application . Therefore, it is arguably the most convenient suture for studying interactions between mechanical tensile stress and MSC differentiation. The increasing evidence that mechanical stimulation is a regulator for osteogenic differentiation in MSCs holds important consequences for the development of orthopedic tissue engineering solutions. The suture stem cells could be an ideal cell type for cell-based craniofacial bone therapy as they possess abilities to differentiate into skeletogenic cell types, generate bones and enhance repair processes. Thus, further investigation is necessary to better understand the molecular mechanism underlying the effects of mechanical stimulation on the osteogenic differentiation of human MSCs in craniofacial sutures.

In conclusion, our results show that cells isolated from the mid-palatal suture of mice demonstrate: MSC characteristics in vitro and express Gli1 markers. These cells when subjected to cyclic tensile strain (aka expansive forces) will demonstrate an upregulation of osteogenic gene expression and an increase in production of mineralized matrix and calcium deposit.

BIBLIOGRAPHY

1. Hou, B., N. Fukai, and B.R. Olsen: *Mechanical force-induced midpalatal suture remodeling in mice*. Bone, 2007. **40**(6): p. 1483-93.
2. SM, A.: *Cellular response to force application at craniofacial sutures*. Orthod Craniofacial Res, 2006. **9**: p. 111-122.
3. Suria L. , T.P.: *Surgically assisted rapid palatal expansion: A literature review*. American Journal of Orthodontics and Dentofacial Orthopedics, 2008. **133**(2): p. 290-302.
4. Liu S.X., Zou, W.: *Effects of rapid maxillary expansion on the midpalatal suture: a systematic review*. European Journal of Orthodontics, 2015: p. 651-655.
5. Proffit, W.R.F., Henry W., Sarver, David M.: *Contemporary Orthodontics, 5e*. 2013.
6. Caprioglio, A.F., R.; Zecca, P.A: International Journal of Molecular Sciences, 2017. **18**: p. 615.
7. Eichenberger, M. and S. Baumgartner: *The impact of rapid palatal expansion on children's general health: a literature review*. Eur J Paediatr Dent, 2014. **15**(1): p. 67-71.
8. Ozan, F.: *Effect of Royal Jelly on new bone formation in rapid maxillary expansion in rats*. Medicina Oral Patología Oral y Cirugía Bucal, 2015: p. e651-e656.
9. Takenouchi, H., et al.: *Longitudinal quantitative evaluation of the mid-palatal suture after rapid expansion using in vivo micro-CT*. Arch Oral Biol, 2014. **59**(4): p. 414-23.
10. Weissman, I.L.: *Stem Cells: Units of Development, Units of Regeneration, and Units in Evolution*, 2000. **100**: p. 157-168.
11. Yen, A.H. and P.T. Sharpe: *Stem cells and tooth tissue engineering*. Cell Tissue Res, 2008. **331**(1): p. 359-72.
12. Fuchs E, S.J.A.: *Stem cells: A new lease on life*. Cell, 2000. **100**: p. 143-155.
13. A. J. Friedensteirn., K.C.K.S.L.: *The Development of Fibroblast Colonies in Monolayer Cultures of Guinea-pig Bone Marrow and Spleen Cells*. Cell Tissue Kinet, 1970. **3**: p. 393-403.

14. Wiesmann, A.: *Decreased CD90 expression in human mesenchymal stem cells by applying mechanical stimulation*. Head Face Med, 2006. **2**: p. 8.
15. Egusa, H.: *Stem cells in dentistry--part I: stem cell sources*. J Prosthodont Res, 2012. **56**(3): p. 151-65.
16. Horwitz, E.M.: *Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement*. Cytotherapy, 2005. **7**(5): p. 393-5.
17. Lee, J.H., H.K. Park, and K.S. Kim: *Intrinsic and extrinsic mechanical properties related to the differentiation of mesenchymal stem cells*. Biochem Biophys Res Commun, 2016. **473**(3): p. 752-7.
18. Discher, D.E., D.J. Mooney, and P.W. Zandstra: *Growth factors, matrices, and forces combine and control stem cells*. Science, 2009. **324**(5935): p. 1673-7.
19. Zhao, H.: *The suture provides a niche for mesenchymal stem cells of craniofacial bones*. Nat Cell Biol, 2015. **17**(4): p. 386-96.
20. Maruyama, T.: *Stem cells of the suture mesenchyme in craniofacial bone development, repair and regeneration*. Nat Commun, 2016. **7**: p. 10526.
21. Spasic, M., K. Maki, and C.R. Jacobs: *Effects of Mechanical Stress on Cells in Comprehensive Biomaterials II*. 2017. p. 102-114.
22. Knapik, D.M.: *Mechanotransduction in bone health, trauma and inflammation*. Antioxid Redox Signal, 2014. **20**(6): p. 970-85.
23. Mao, J.J.: *Mechanobiology of Craniofacial Sutures*. J Dent Res, 2002. **81**(12): p. 810-816.
24. Steward, A.J. and D.J. Kelly: *Mechanical regulation of mesenchymal stem cell differentiation*. J Anat, 2015. **227**(6): p. 717-31.
25. Carroll, S.F., C.T. Buckley, and D.J. Kelly: *Cyclic Tensile Strain Can Play a Role in Directing both Intramembranous and Endochondral Ossification of Mesenchymal Stem Cells*. Front Bioeng Biotechnol, 2017. **5**: p. 73.
26. Hao, J.: *Mechanobiology of mesenchymal stem cells: Perspective into mechanical induction of MSC fate*. Acta Biomaterialia, 2015. **20**: p. 1-9.

27. Lohberger, B.: *Effect of cyclic mechanical stimulation on the expression of osteogenesis genes in human intraoral mesenchymal stromal and progenitor cells.* Biomed Res Int, 2014. **2014**: p. 189516.
28. Jang, J.Y.: *Combined effects of surface morphology and mechanical straining magnitudes on the differentiation of mesenchymal stem cells without using biochemical reagents.* J Biomed Biotechnol, 2011. **2011**: p. 860652.
29. Liu, Y.: *Suture cartilage formation pattern varies with different expansive forces.* Am J Orthod Dentofacial Orthop, 2014. **146**(4): p. 442-50.
30. Katebi, N.: *The mouse palate and its cellular responses to midpalatal suture expansion forces.* Orthod Craniofac Res, 2012. **15**(3): p. 148-58.
31. E.T.Kobayashi, F.H., Y.Kobayashi,E.Sakai,Y.Miyazaki,T.Kamiya,K.Kobayashi, Y.Kato,andH.Sakai: *Force-induced Rapid Changes in Cell Fate at Midpalatal Suture Cartilage of Growing Rats.* J Dent Res 1999. **78**(9): p. 1495-1504.
32. I. Takahashi, I.M., M. Nakamura, Y. Sasano, S. Saitoh, I M. Kagayama, and H. Mitani J: *Effects of Expansive Force on the Differentiation of Midpalatal Suture Cartilage in Rats.* Bone, 1996. **18**(4): p. 341-348.
33. Zahrowski JJ, T.P.: *Force magnitude effects upon osteoprogenitor cells during premaxillary expansion in rats.* Angle Orthod, 1992. **62**(3): p. 197-202.
34. Akhouayri O, L.-P.M., Rattner A, Laroche N, Caillot- Augusseau A, Alexandre C, Vico L: *Effects of static or dynamic mechanical stresses on osteoblast phenotype expression in three-dimensional contractile collagen gels* J Cell Biochem 1999. **76**: p. 217-30.
35. Mullender M, E.H.A., Yang Y, van Duin MA, Burger EH, Klein- Nulend J: *Mechanotransduction of bone cells in vitro: mech- anobiology of bone tissue.* Med Biol Eng Comput, 2004. **42**: p. 14-21.
36. Zhang, P.: *Osteogenic response of mesenchymal stem cells to continuous mechanical strain is dependent on ERK1/2-Runx2 signaling.* Int J Mol Med, 2012. **29**(6): p. 1083-9.
37. Friedl, G.: *Undifferentiated human mesenchymal stem cells (hMSCs) are highly sensitive to mechanical strain: transcriptionally controlled early osteochondrogenic response in vitro.* Osteoarthritis Cartilage, 2007. **15**(11): p. 1293-300.

38. Jacobs, J.C.C.a.C.R.: *Mechanically induced osteogenic lineage commitment of stem cells*. Chen and Jacobs Stem Cell Research & Therapy 2013. **4**(107): p. 1-10.
39. Ward, D.F., Jr.: *Mechanical strain enhances extracellular matrix-induced gene focusing and promotes osteogenic differentiation of human mesenchymal stem cells through an extracellular-related kinase-dependent pathway*. Stem Cells Dev, 2007. **16**(3): p. 467-80.
40. Simmons, C.A.: *Cyclic strain enhances matrix mineralization by adult human mesenchymal stem cells via the extracellular signal-regulated kinase (ERK1/2) signaling pathway*. J Biomech, 2003. **36**(8): p. 1087-96.
41. Bancroft, G.N.: *Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner*. Proc Natl Acad Sci U S A, 2002. **99**(20): p. 12600-5.
42. Shi, Y.: *Gli1 identifies osteogenic progenitors for bone formation and fracture repair*. Nat Commun, 2017. **8**(1): p. 2043.